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# Regeneration of *Acacia melanoxylon* plantlets *in vitro*

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Callus from juvenile material of mature *Acacia melanoxylon* R. Br. trees formed shoots on a Murashige and Skoog's medium when supplemented with benzyladenine (1μM) and indole-3-acetic acid (1 μM). Indole-3-butyric acid at 10 μM was the best treatment for the induction and growth of roots on the *in vitro*-produced shoots.

Kallus wat gevorm is deur jeugdige materiaal van *Acacia melanoxylon* R. Br. het lote gevorm op 'n medium van Murashige en Skoog in die teenwoordigheid van bensieladenien (1 μM) en indool-3-asynsuur (1 μM). Die optimale behandeling vir die induksie van wortels op die *in vitro*-gekweekte lote was indool-3-bottersuur teen 'n konsentrasie van 10 μM.

**Keywords:** *Acacia melanoxylon, in vitro* culture, rooting shoot formation

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## Introduction

Relatively few woody species of the Leguminosae have been cultured *in vitro*. Examples of successful cultured plants are *Acacia koa* Gray (Skolmen & Mapes 1976), *Dalbergia sissoo* Roxbg. (Mukhopadhyay & Mohan Ram 1981), *Prosopis cineraria* Linn. (Goyal & Arya 1981) and *Albizzia lebbeck* L. (Gharyal & Maheshwari 1983). The Australian introduced species *Acacia melanoxylon* R. Br. (Blackwood) is a source of highly valued and popular furniture timber in the Republic of South Africa. To produce high quality timber on a large scale, large numbers of cloned plants from selected trees must be produced for afforestation purposes. The large-scale production of clones can be achieved through *in vitro* culture techniques. In this communication we report on the *in vitro* regeneration of *Acacia melanoxylon* plantlets from mature trees.

## Materials and Methods

Explants were taken from mature branches of 5-m high *Acacia melanoxylon* trees. Juvenile explant material was taken from stump coppice. This material was characterized by the presence of compound feathered true leaves. In adult material the feathered leaves were absent. The shoots collected were green, soft and from the present season's growth. Explant material was only collected from October to February during the active growing season. The collected branches were thoroughly washed with water and soap. They were then surface sterilized in 0,2% mercuric chloride for 20 min followed by five rinses with sterilized water. Explants consisted of an axillary bud and a 5-mm section of the shoot. The explants were inoculated under aseptic conditions onto the nutrient medium. The nutrient medium of Murashige and Skoog (1962) (MS) was used with 2% sucrose and 0,8% Difco agar. The pH of the medium was adjusted to 5,7 before autoclaving at 121°C for 20 min. Growth regulators used for the induction of shoots were benzyladenine (BA) at concentrations of 1, 5 and 10 μM in a 3 × 4 factorial experiment with indole-3-acetic acid (IAA) at concentrations of 0, 1, 5 and 10 μM. This experiment consisted of three replications and 10 explants per treatment. The rooting experiment consisted of treatments with 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and IAA each at concentrations of 0, 1, 5 and 10 μM. Thirty explants were used for treatment. The growth regulators were autoclaved, except IAA which was filter sterilized with a 0,22 μm filter. Nutrient medium (10 $cm^3$) in 2,5 cm × 9 cm glass vials was used for the culture of an explant. The cultures were incubated at 25 ± 2°C in continuous light of *ca.* 5 $Wm^{-2}$ irradiance (400 – 700 nm).

Differences between treatment means were determined by

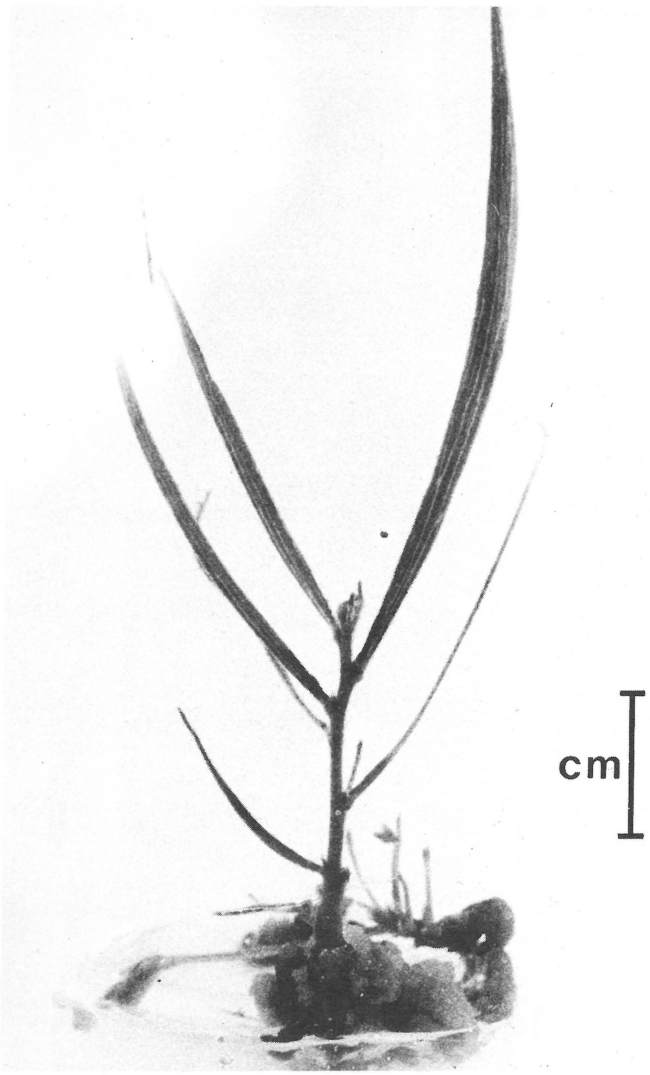


**Figure 1** Mature shoot of *Acacia melanoxylon* formed *in vitro* from a mature axillary bud explant.

Duncan's multiple range test at a 90% level of confidence (Steel & Torrie 1960).

## Results and Discussion

After 60 days of culture the juvenile axillary bud explants produced 1 – 2-cm long shoots with normal juvenile leaves. The axillary buds of these shoots elongated within 30 days to form a multiple-shoot culture with three to seven shoots per explant. The reaction of the explant was optimal in the presence of BA (1 μM) in combination with IAA (1 – 5 μM). Colourless friable callus developed in the presence of BA (1 μM) with IAA (10 μM) and BA (5 – 10 μM). The juvenile explants did not react satisfactorily on the treatment without IAA.

The mature axillary bud explants were not as responsive as the juvenile explants. Axillary buds formed 0,5 – 1-cm mature shoots (Figure 1) but the axillary buds of these shoots stayed dormant. The growth response was precarious and not more than 35% of the explants responded to the growth regulator treatment. The elongation of phyllodes without shoot growth occurred sporadically in most treatments. Colourless friable callus developed on BA (5 – 10 μM) in combination with IAA (5 – 10 μM).

In a subsequent experiment 40 1-cm long juvenile shoot segments without axillary buds were incubated on a medium with the same treatments as described above. In the presence of BA (1 – 5 μM) without IAA, 40% of the explants developed one to three shoots at the wounded areas. These shoots were normal with normal juvenile leaves. The shoots were 3 – 4 cm long (Figure 2), grew more vigorously and were longer than the shoots which developed from explants with axillary buds. The axillary buds of these shoots remained dormant. The results indicated that morphogenesis and normal shoot development can be achieved in the absence of apical meristematic tissue.

To determine whether phyllodes were also morphogenic an experiment was conducted with phyllode explants using similar treatments as described above. Phyllode explants (1 × 0,5 cm) were dissected after surface sterilization. After 60 days of culture the phyllodes had formed callus on all the treatments except in the absence of IAA. After 30 to 40 days two to six shoots had developed per explant in the presence of BA (1 μM) in combination with IAA (0 – 5 μM). The shoots were 0,3 to 0,5 cm long and vitreous in appearance. Abnormally small leaves developed on the shoots. Morphogenesis was sporadic and only 35% of the explants responded to the best treatment. Despite the poor shoot development and low quality of the shoots the results show that morphogenesis is possible from phyllodes.

From the results of the first experiment with juvenile axillary bud explants, BA (1 μM) in combination with IAA (1 μM) was selected as the best treatment for subsequent experiments with axillary bud explants. Shoots (2 – 3 cm long), which had developed from the shoots of the axillary bud explants were put on a medium with BA (1 μM) in combination with IAA (1 μM). Within 40 days callus formed at the base of the shoots and multiple buds developed on the callus (Figure 3). After 60 days of culture 64% of the buds elongated to form normal shoots.

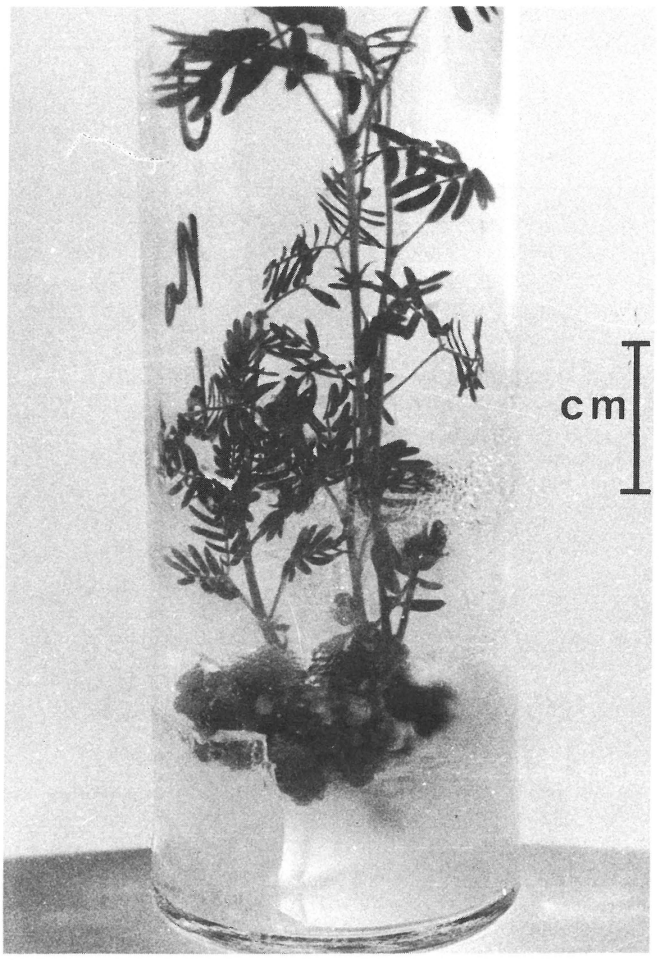


**Figure 2** Shoots formed at the cut edge of a juvenile shoot explant of *Acacia melanoxylon*. The explant had no axillary buds.

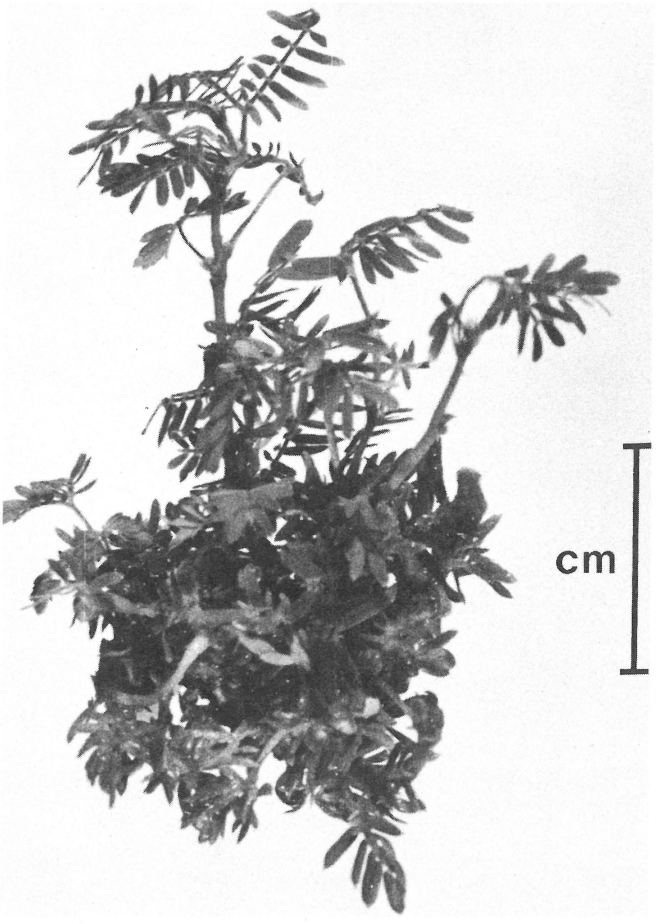


**Figure 3** Multiple buds formed on callus from juvenile shoot explants of *Acacia melanoxylon*.

## Rooting of shoots

Shoots (1 – 5 cm long) were taken from the multiple bud cultures and placed on nutrient medium with NAA, IBA and IAA at concentrations of 0, 1, 5 and 10 µM each. Only 5% of the shoots shorter than 2 cm rooted. No shoots rooted in the absence of auxin and only 5% rooted in the presence of 1 µM auxin. To determine the efficiency of the root-promoting treatments various parameters were measured. Firstly the rooting of shoots after different periods of incubation was determined. From the results in Table 1 it is clear that the highest percentage of shoots (82%) rooted on NAA (5 µM) in the shortest period of time (40 days). Similar values for the rooting of shoots were obtained with IBA and IAA at 10 µM but only after 60 days of incubation. It was also noted that after 20 days of incubation very few additional shoots rooted on IBA and IAA at 5 µM. The same phenomenon occurred on NAA (5 µM) after 40 days of incubation. Shoots treated with the three auxins at 10 µM however, still rooted at the termination of the experiment after 60 days (Table 1).

Secondly, the mean number of roots formed per shoot and the mean length of the roots were determined for each treatment. No difference was recorded in the length of the roots formed by the shoots with the different treatments. From the results outlined in Table 2 it is evident that significantly more roots were initiated per shoot in the presence of IBA (10 µM) than with the other treatments.

Thirdly, the mean number and mean length of the secondary roots formed was determined. It is clear from Table 3 that appreciably more secondary roots were formed on the medium with IBA (10 µM). Indole-3-butyric acid at a concentration of 5 µM proved to be the best treatment to stimulate secondary root growth (Table 4 and Figure 4).

From the results on the rooting of the shoots it can be concluded that IBA was the best promoter of primary and secondary root induction and secondary root growth. The induction of roots are more important in this first stage of root development than elongation since it only takes place in the presence of applied auxins while root elongation takes place in the absence of applied auxins. In view of this it appears that IBA at a concentration of 10 µM is the appropriate treatment for the rooting of shoots.

**Table 1** The effect of auxins on the rooting of *Acacia melanoxylon* shoots *in vitro* over a period of 60 days. Percentage rooting is indicated in the Table

| Incubation period (days) | Auxin and concentration (µM) | | | | | |
|---|---|---|---|---|---|---|
| | NAA | | IBA | | IAA | |
| | 5 | 10 | 5 | 10 | 5 | 10 |
| 10 | 8,3 | 0 | 2,0 | 13,3 | 21,6 | 31,6 |
| 20 | 35,0 | 23,0 | 51,6 | 55,0 | 51,6 | 53,3 |
| 40 | 81,7 | 50,5 | 51,6 | 71,6 | 53,3 | 76,0 |
| 60 | 83,3 | 56,6 | 51,6 | 81,7 | 53,3 | 81,7 |

**Table 2** The effect of NAA, IBA and IAA at different concentrations on the number of roots formed on shoot explants of *Acacia melanoxylon* over 60 days of *in vitro* culture (mean ± standard error)

| | Auxin and concentration (µM) | | | | | |
|---|---|---|---|---|---|---|
| | NAA | | IBA | | IAA | |
| | 5 | 10 | 5 | 10 | 5 | 10 |
| Number of roots formed per shoot | 3,1 ± 0,6 | 2,4 ± 0,7 | 2,3 ± 0,7 | 4,8 ± 0,6 | 2,5 ± 0,6 | 2,0 ± 0,7 |

**Table 3** The effect of NAA, IBA and IAA at different concentrations on the number of secondary roots formed per cm of adventitious root length of *Acacia melanoxylon* shoot explants after 60 days of *in vitro* culture (mean ± standard error)

| | Auxin and concentration (µM) | | | | | |
|---|---|---|---|---|---|---|
| | NAA | | IBA | | IAA | |
| | 5 | 10 | 5 | 10 | 5 | 10 |
| Number of secondary roots formed per cm of adventitious root | 2,7 ± 1,2 | 0,8 ± 0,6 | 2,0 ± 0,8 | 6,2 ± 1,4 | 2,4 ± 0,7 | 2,8 ± 0,9 |

**Table 4** The effect of different concentrations of NAA, IBA and IAA on the growth of secondary roots in shoot cultures of *Acacia melanoxylon* after 60 days of *in vitro* incubation (mean length of secondary roots ± standard error)

| | Auxin and concentration (µM) | | | | | |
|---|---|---|---|---|---|---|
| | NAA | | IBA | | IAA | |
| | 5 | 10 | 5 | 10 | 5 | 10 |
| Length of secondary roots (mm) | 4,2 ± 1,8 | 6,2 ± 2,0 | 11,1 ± 2,1 | 5,1 ± 1,9 | 4,3 ± 1,4 | 7,5 ± 2,0 |

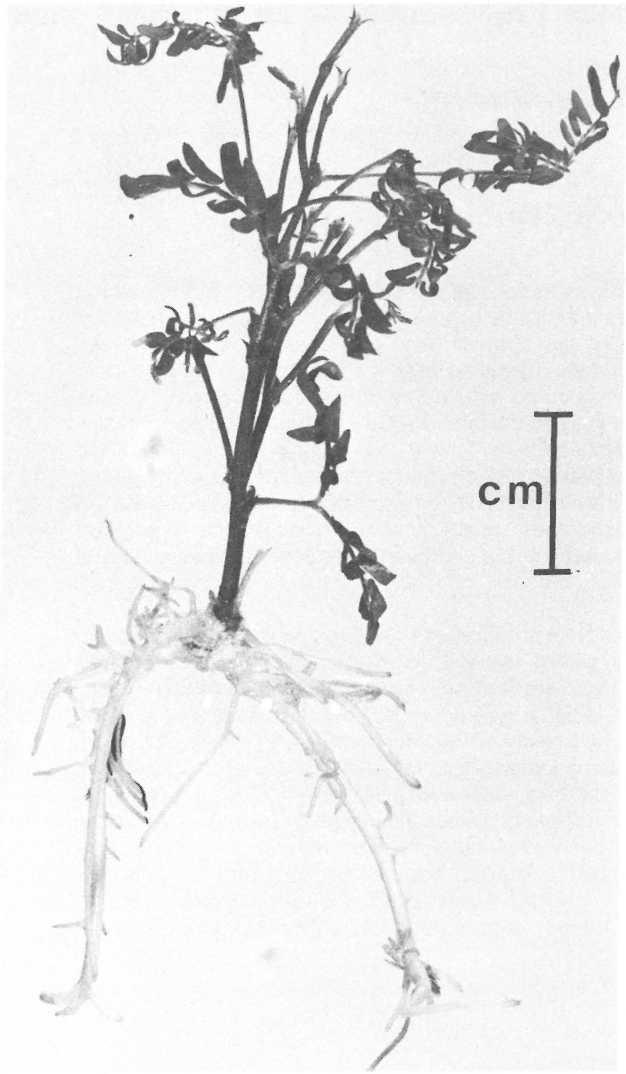


**Figure 4** Juvenile plantlet of *Acacia melanoxylon* cultured *in vitro*.

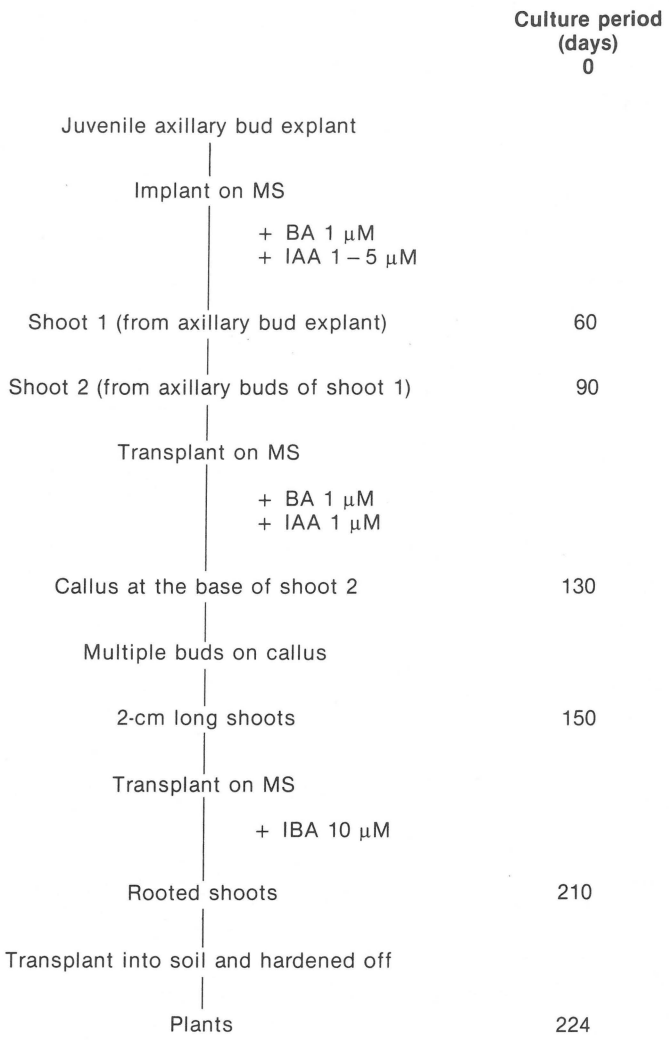


**Figure 5** Flow diagram to illustrate the *in vitro* culture of *Acacia melanoxylon* from axillary bud explants.

## Hardening off of plants

The rooted shoots were removed from the medium after 60 days of incubation on the rooting medium. The agar was washed from the roots with tap water. Two methods were used to harden off the plantlets. In the first instance 25 plantlets were planted in autoclaved soil in pots with a diameter of 8 cm and height of 10 cm. After the plants had been watered each plant was covered with a plastic bag and kept under similar conditions as the *in vitro* cultures. The plants were very sensitive to the ambient humidity and if exposed for a few minutes to the environment after being removed from the culture tubes, they lost their leaves as a result of desiccation. The potted plants were kept covered with plastic bags for 2 weeks. After this period the humidity inside the bags was reduced by increasing the perforation of the plastic bags over a period of 30 days. Seventy-two percent of the plants survived this treatment.

Secondly, plantlets were hardened off in a mist bed. Forty plantlets were planted in autoclaved sand with a particle size of 0,71 to 1,00 mm. The plantlets were maintained in a fibre glass-enclosed mistbed and received six periods each of 15 sec mist per hour. The minimum temperature was 4°C and the maximum temperature 23°C during the hardening off period. Hardening off was achieved within 2 weeks. Ninety percent of the plants survived. The latter hardening off procedure was less labour intensive and more successful.

It can be concluded from the results that *Acacia melanoxylon* can be successfully cultured *in vitro* by using the procedure as is summarized in Figure 5. The cloning of mature *Acacia melanoxylon* trees *in vitro* is a valuable method for the mass production of selected trees for high quality furniture timber production.

## Acknowledgements

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